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EXHIBIT 8

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Immunology

Serological analysis of cell surface antigens of null cell acute lymphocytic leukemia by mouse monoclonal antibodies

(human leukemia/hybridoma/leukemia-specific antigens/differentiation antigens)

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ABSTRACT Nine antigen systems were defined. Two were related to HLA-A,B,C and to Ia-like antigens; the others could be grouped into three categories. (*i*) NL-22, NL-1: NL-22 antibody reacted with leukemia cells from 12 of 16 cases of null cell acute lymphocytic leukemia (null-ALL) but not with any other type of leukemia tested or with lymphoid cells of various origins. Among cultured cell lines tested, one (NALM-6) of three null-ALL cell lines was positive; the others were negative. Absorption analysis confirmed the restriction of NL-22 antigen to null-ALL. NL-1 antibody was reactive with leukemia cells from 10 of 16 cases of null-ALL and 3 of 6 cases of chronic myelocytic leukemia in blastic crisis (CML-BC). The antigen was present also on a minor population of normal lymphoid cells. The distribution and molecular weight (100,000; glycoprotein) of the NL-1 antigen resembled that of the previously described common ALL antigen (cALL). (*ii*) NL-30, NL-4: Both antibodies exhibited almost identical patterns of reactivities against cultured cell lines tested. They reacted with leukemia cells from some cases of null-ALL, adult T-cell leukemia, and CML-BC, although they showed discordance in their reactivities against a panel of leukemia cells. (*iii*) NL-9, NL-8, NL-25: These three antibodies detect serologically distinguishable determinants on a broad range of leukemias and normal lymphoid and hematopoietic cell types. The antibodies analyzed in this study provide evidence for the heterogeneity of null-ALL by demonstrating a variety of antigen phenotypes on leukemia cells. One of the antigens (NL-22) appears to be restricted to null-ALL.

By using conventional heteroantisera, the cell surface antigens of erythrocyte rosette-negative (E^-), surface immunoglobulin-negative (sIg^-) acute lymphocytic leukemia (null-ALL) have been studied in considerable detail (1–5). One of the best characterized antigens of null-ALL is the common ALL antigen (cALL) (6), which was first reported to be present only in null-ALL and chronic myelocytic leukemia in blastic crisis (CML-BC) but was later shown to be also expressed by a small fraction of fetal liver and regenerating bone marrow cells (7). Biochemical characterization of the cALL showed it to be a M_r 100,000 glycoprotein (gp100) (8). Several other laboratories have also detected gp100 antigens on ALL cells with similar serological characteristics (3, 9, 10).

In the present study, monoclonal antibodies were developed against null-ALL cells obtained from a single patient, and nine distinct antigen systems were defined. Among these, the NL-22 antigen is restricted to null-ALL and the NL-1 antigen closely resembles cALL. Surface antigenic profiles of leukemia cells from 16 cases of null-ALL were studied by using this panel of monoclonal antibodies.

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MATERIALS AND METHODS

Leukemia and Lymphoid Cells. Peripheral blood or bone marrow aspirates obtained from patients with leukemia or normal volunteers were separated by Ficoll/Paque (Pharmacia) density sedimentation (11), and interface layers were collected. Aliquots of the cell suspensions were cryopreserved in liquid nitrogen. Specimens of lymphoid tissues (tonsil, spleen, and fetal thymus) were placed in RPMI-1640 (GIBCO) containing 10% (vol/vol) fetal bovine serum (Microbiological Associates, Walkersville, MD). Single-cell suspensions were obtained by gentle manual squeezing of tissue specimens in sterile vinyl bags.

Cell surface markers—surface immunoglobulin (sIg), sheep erythrocyte rosettes (E), erythrocyte–antibody–complement rosettes (EAC), and erythrocyte–antibody rosettes (EA)—were determined by K. Koike and T. Suchi of this institution, according to the method of Tachibana and Ishikawa (12).

Cultured Cell Lines. Cultured cell lines derived from hematopoietic tumors and other malignancies are listed in Table 1 (see refs. 13–16). Cultures were maintained in RPMI-1640 supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), 2 mM glutamine and 10% fetal bovine serum.

Serological Procedure. Immune adherence assays and qualitative absorption tests were performed as described (15, 16). When single-cell suspensions were used as target cells, cells were washed twice with RPMI-1640 and 5,000 cells in 10 μl of RPMI-1640 was seeded into each well of Falcon 3034 plates. Plates were incubated for 2–3 hr in 5% CO_2 in air in a humidified incubator, washed, and then used for immune adherence assays.

Production of Hybridomas Against 063 Null-ALL Cells. 063 ALL cells were obtained from the peripheral blood of a 22-year-old female patient. The surface phenotype of the leukemia cells was E (2%), EAC (36%), EA (4%), and sIg (0%). A BALB/c female mouse was immunized three times at 4-week intervals with 10^7 leukemia cells. The first immunization was with fresh leukemia cells injected subcutaneously, the second immunization was with cryopreserved cells injected subcutaneously, and the third injection was with cryopreserved cells injected intraperitoneally. The mouse was sacrificed 4 days after the last immunization and spleen cells were obtained for hybridization.

Cell hybridization was performed according to the method

Abbreviations: E, sheep erythrocyte rosette; sIg, surface immunoglobulin; ALL, acute lymphocytic leukemia; null-ALL, null cell ALL; T-ALL, T cell ALL; B-ALL, B cell ALL; CML-BC, chronic myelocytic leukemia in blastic crisis; cALL, common ALL antigen; EA, erythrocyte–antibody rosette; EAC, erythrocyte–antibody–complement rosette; ATL, adult T-cell leukemia; B-CLL, B-cell chronic lymphocytic leukemia; AML, acute myelocytic leukemia.

of Köhler and Milstein (17). Spleen cells (2×10^8) were fused with 4×10^7 MOPC-21 NS/1 cells in 0.2 ml of 42% (wt/vol) polyethylene glycol (M_r 4,000; Koch-Light Laboratories, Bucks, England) in phosphate-buffered saline with 15% (vol/vol) dimethyl sulfoxide (Merck, Darmstadt, Federal Republic of Germany). Selection of hybrids in hypoxanthine/aminopterin/thymidine (HAT) medium and cloning by the limiting dilution method were performed as described (16).

Immunoprecipitation Procedure. Raji cells were metabolically labeled with [^{3}H]glucosamine, and immunoprecipitation was carried out as described (16).

RESULTS

Reactivity of Monoclonal Antibodies with Cultured Tumor Cell Lines. Hybridoma colonies were observed in 180 of 960 wells and antibody activity against the surface antigens of the immunizing null-ALL cell was demonstrated in 32 supernatants by immune adherence assays. Screening of these antibodies against a panel of 21 cell lines classified their reactivity into nine groups (Table 1). One antibody from each group was selected for further serological analysis. Seven of the nine antibodies were of the IgM class, and the two others (NL-1 and NL-12) were γ2a. Because immune adherence assays detect complement-fixing antibodies, this distribution of immunoglobulin classes is what one might expect.

In tests on the cultured tumor cell lines, NL-22 antibody showed the most restricted activity. Only one cell line, NALM-6 derived from null-ALL, reacted weakly with NL-22 antibody. NL-1 antibody was strongly reactive with two null-ALL cell lines (NALL-1, NALM-6), the two Burkitt lymphoma cell lines, and one DHL cell line but not with a number of other cell lines. NL-30 and NL-4 antibodies, with a single exception, showed the same pattern of reactivity on the panel of cultured cells. However, tests with a number of different leukemias (see below) indicated that these two antibodies detected different antigenic determinants. The antigen detected by NL-9 antibody was strongly represented on T-cell lines and on K-562 but was not detected on the nonhematopoietic cell lines. NL-8 and NL-25 antibodies reacted with a broader range of cells. They can be distinguished by their patterns of reactivity, particularly by the fact that NL-25 antibody did not react with sIg$^+$ cell types. NL-12 antibody reacted with all B-cell lines tested and with the SK-MEL-37 melanoma cell line, which is known from past work to express Ia-like antigens (18). NL-19 antibody had strong reactivity with all cell types tested except Daudi, K-562, and SK-MEL-33. Because these cell lines lack HLA-A, B, C expression, it appears likely that NL-19 detects a HLA-related determinant.

Reactivity of Monoclonal Antibodies with Lymphoid and Hematopoietic Cell Populations. Eight monoclonal antibodies were tested against peripheral blood lymphocytes and lymphoid and hematopoietic cell populations from various sources (Table 2). NL-22 antibody did not react with any of the cell populations. NL-1 antibody was reactive with 0–10% of cells derived from fetal thymus, bone marrow, and non-T peripheral blood lymphocytes but not with any of the other lymphoid cells tested. NL-30 and NL-4 antibodies also showed relatively restricted reactivity—e.g., 5–40% of bone marrow cells and 0–10% of fetal thymocytes. NL-9, NL-8, NL-25, and NL-12 antibodies

Table 1. Titers of nine monoclonal anti-ALL (063) antibodies against cultured cell lines determined by immune adherence assay

Cell line	Origin	Surface marker; %				Monoclonal antibodies, titer × 10^{-3}								
		E	EAC	EA	sIg	NL-22 (μ)	NL-1* (γ2a)	NL-30 (μ)	NL-4 (μ)	NL-9 (μ)	NL-8 (μ)	NL-25 (μ)	NL-12* (γ2a)	NL-19 (μ)
Immunizing cell														
063	null-ALL	2	36	4	—	30	10	25	25	100	25	125	10	300
Non-T, non-B														
NALM-16	null-ALL	0	0	0	—	—	—	—	—	—	—	125	—	125
NALL-1	null-ALL	0	18	5	—	—	1	—	—	—	100	600	1	15,000
NALM-6	null-ALL	6	0	1	—	0.2	10	—	—	1	25	100	5	15,000
NALM-1	CML-BC	2	0	2	—	—	0.2	—	—	—	25	600	5	3,000
K-562	CML-BC	0	0	91	—	—	—	—	—	100	25	—	—	—
T cell														
CCRF-CEM	T-ALL	0	0	25	—	—	0.4	—	—	15,000	—	600	—	80,000
CCRF-HSB-2	T-ALL	0	3	1	—	—	—	—	—	1	—	25	—	600
RPMI 8402	T-ALL	0	10	28	—	—	0.9	—	—	25	—	3,000	—	15,000
MOLT-3	T-ALL	0	7	25	—	—	—	—	—	3,000	—	600	—	15,000
P-12	T-ALL	88	93	0	—	—	—	0.2	0.2	600	1	10,000	—	50,000
MT-1	ATL	0	67	1	—	—	—	—	—	—	25	600	—	1,000
B cell														
Raji	Burkitt†	0	91	23	—	—	5	—	—	—	—	100	25	15,000
Daudi	Burkitt	0	97	89	μκ	—	1	—	—	—	—	—	5	—
CCRF-SB	B-ALL‡	0	51	4	γκ	—	—	—	—	0.2	25	—	25	100
BALL-1	B-ALL	0	0	47	μδλ	—	—	—	—	—	—	—	25	3,000
SK-DHL-2	DHL§	0	10	0	μλ	—	1	—	—	—	—	—	25	5×10^6
RPMI 8226	Myeloma	0	0	0	λ	—	—	—	—	—	25	—	100	15,000
Solid tumor														
SK-MEL-37	Melanoma					—	—	5	1	—	25	25	5	25
SK-MEL-33	Melanoma					—	—	5	5	—	100	100	—	—
SK-N-SH	Neuroblastoma					—	—	5	5	—	100	600	—	100
SK-RC-7	Renal ca					—	—	—	1	—	25	25	—	100

* Both NL-1 and NL-12 antibodies can be detected by protein A assays, and the titers detected are generally 100–1,000 times higher than those by immune adherence assays shown in this table.
† Burkitt lymphoma.
‡ B-ALL, B cell ALL.
§ DHL, diffuse histiocytic lymphoma.

Table 2. Immune adherence reactivity of eight monoclonal anti-ALL (063) antibodies tested against various lymphoid and hematopoietic cells

Test cell	n*	% positive cells at 1:200 dilution							
		NL-22	NL-1	NL-30	NL-4	NL-9	NL-8	NL-25	NL-12
Fetal thymocytes†	7	<3	0–10	0–10	0–10	0–10	40–70	40–80	5–20
Peripheral blood lymphocytes	14								
T fraction		<3	<3	<3	0–10	<3	20–50	40–70	5–20
Non-T fraction		<3	0–10	<3	<3	0–10	40–70	0–10	30–80
Spleen cells‡	8	<3	<3	<3	0–10	20–70	20–70	20–60	20–70
Tonsil cells	4	<3	<3	<3	<3	0–10	20–50	0–10	50–70
Bone marrow cells§	7	<3	0–10	5–40	5–20	30–70	20–50	20–70	10–30

* Number of samples tested.
† Obtained from a fetus at second to third trimester. Fetal thymocytes were studied because adult thymocytes were not available.
‡ Obtained from patients with gastric cancer who underwent gastrosplenectomy.
§ Obtained from patients with idiopathic thrombocytopenic purpura.

reacted with a broad range of lymphoid and hematopoietic cell types.

Reactivity of Monoclonal Antibodies with Leukemia Cells of Various Types. Leukemia cells from 51 patients, including 16 with null-ALL, were collected and tested as either fresh or frozen cells for reactivity with the eight monoclonal antibodies (Table 3). Interestingly, NL-22 antibody did not react with all null-ALL cells; 4 of the 16 cases of null-ALL were negative. No other leukemia cell types, including T cell ALL (T-ALL), adult T-cell leukemia (ATL) (19), B-cell chronic lymphocytic leukemia (B-CLL), and acute myelocytic leukemia (AML), reacted with NL-22 antibody. The reactivity of NL-1 antibody was generally restricted to null-ALL and CML-BC. Both NL-30 and NL-4 antibodies also reacted mainly with ALL and CML-BC, although they were reactive with some cases of ATL. On the other hand, NL-9, NL-8, and NL-25 antibodies reacted with AML as well as ALL. The Ia-like antigen defined by NL-12 antibody (see below) was detected on a majority of the leukemias of all types tested. Typing of 16 individual null-ALL cases with the eight monoclonal antibodies is summarized in Table 4. These tests demonstrated considerable heterogeneity in the cell surface phenotype of null-ALL from different patients.

Qualitative Absorption Analysis of NL-22 and NL-1 Antibodies. To confirm the specificity of NL-22 antibody for null-ALL cells, absorption analysis was carried out with 063 null-ALL as target cells. Two of the three null-ALL cell lines tested, NALM-6 and NALL-1, absorbed immune adherence reactivity from NL-22 antibody (Table 5). The 19 other cell lines studied showed no absorption. In agreement with the results of direct tests, null-ALL cells from seven of nine cases removed reactivity. No other normal or leukemic cell type removed NL-22 antibody reactivity. Thus, both direct tests and absorption analysis indicate that the antigen detected by NL-22 antibody is restricted to null-ALL cells.

Absorption analysis of NL-1 antibody was also carried out using 063 null-ALL as target cells (Table 6). Cultured cell lines reacting with NL-1 antibody in direct tests (Table 1) removed NL-1 reactivity in absorption tests. In tests with leukemia cells, four of five null-ALL cases showed positive absorption. These findings also correspond with the result of direct tests. Because NL-1 antibody belongs to the $\gamma 2a$ class, it can be detected also by protein A assays (20). Absorption of NL-1 antibody in these assays gave the same results as absorption analysis using immune adherence assays.

Immunoprecipitation Tests with NL-1 and NL-12 Antibodies. Nonidet P-40 lysates of [^{3}H]glucosamine-labeled Raji cells were immunoprecipitated with NL-1 or NL-12 antibodies and the precipitates were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The antigen detected by NL-1 antibody was a glycoprotein of M_r 100,000. NL-12 antibody precipitated two bands with M_rs of 34,000 and 28,000, characteristic of Ia-like antigens. In accordance with the general experience that IgM monoclonal antibodies fail to immunoprecipitate, attempts to precipitate the antigens identified by NL-22 antibody from radiolabeled extracts of null-ALL cells were unsuccessful.

DISCUSSION

Since the introduction of hybridoma techniques, several groups have attempted to produce monoclonal antibodies with specificity for ALL cells. Ritz *et al.* (21) described a monoclonal antibody (J-5) that appeared to be specific for cALL originally defined by Greaves with conventional heteroantibody. J-5 antibody was initially reported to react only with NALM-1 and LAZ-222 null-ALL cell lines, but subsequent studies showed

Table 3. Immune adherence reactivity of eight monoclonal anti-ALL (063) antibodies tested against leukemias of various types

Leukemia	n*	Cases, no.†							
		NL-22	NL-1	NL-30	NL-4	NL-9	NL-8	NL-25	NL-12
ALL									
null-ALL	16	12	10	5	7	5	13	10	13
T-ALL	4	0	0	0	0	0	2	4	2
ATL	7	0	1	3	2	5	5	5	4
AML	11	0	1	1	0	5	8	5	8
CML-BC	6	0	3	4	2	5	5	5	3
B-CLL	7	0	0	0	0	1	3	0	4

* Number of cases tested.
† Cases that showed >20% positive reaction at a serum dilution of 1:200.

Table 4. Typing of 16 null-ALL cells* with eight monoclonal anti-ALL (063) antibodies

Case	Age, yr; sex	Surface markers, %			Immune adherence reactivity of monoclonal antibodies†							
		E	EAC	EA	NL-22	NL-1	NL-30	NL-4	NL-9	NL-8	NL-25	NL-12
063	23; F	2	36	4	++	++	++	++	+	++	++	++
171	20; F	2	71	35	++	++	+	++	±	++	++	++
343	35; M	3	32	4	+	++	+	+	±	++	+	++
277	45; M	4	66	11	+	++	+	++	−	++	±	++
038	40; F	4	2	3	++	++	−	+	+	+	++	++
059	20; M	3	2	3	++	±	−	±	+	++	+	++
538	18; F	4	0	1	+	+	−	+	−	++	±	++
559	6; M	3	2	0	+	++	−	−	−	+	+	+
537	41; F	1	69	9	+	+	−	−	−	+	+	+
384	13; M	1	6	12	+	+	−	−	±	−	+	++
352	18; M	4	21	11	+	±	−	−	−	+	±	+
041	12; M	2	9	2	+	±	−	−	−	+	+	±
542	40; F	5	12	0	−	++	+	++	±	+	−	++
514	40; M	5	1	4	−	−	−	±	−	−	±	±
040	43; F	2	1	3	−	−	−	−	++	+	+	+
063	8; M	1	2	4	−	−	−	−	+	±	−	−
No. positive:					12	10	5	7	5	13	10	13

* All cells were obtained from peripheral blood except that in cases 559 and 537 they were from bone marrow.
† Reactions obtained at a serum dilution of 1:200 evaluated as following: −, <1%; ±, 1–20%; +, 20–70%; ++, >70%.

that it was reactive with some B-cell lines (22) and also with normal tissues (23). The reactivity of the NL-1 monoclonal antibody defined in this study closely resembles findings with the J-5 antibody. In addition, both J-5 and NL-1 antibodies identified glycoproteins in the M_r 95,000–100,000 range. It appears likely, therefore, that J-5 and NL-1 antibodies detect identical or closely related molecules.

In contrast to the findings with J-5 and NL-1 antibodies, NL-22 antibody identifies an antigen that appears to be restricted to null-ALL cells. Among 21 cell lines tested, only NALM-6 derived from a case of null-ALL, reacts with NL-22 antibody. Leukemia cells from 12 of 16 cases of null-ALL express the antigen detected by NL-22 antibody; no other normal or leukemic cell type was found to react with NL-22 antibody. Absorption analysis with NL-22 antibody confirmed that null-ALL was the only cell type that expressed the NL-22 antigen. Kersey recently described a monoclonal antibody (BA-2) produced against a NALM-6 cell lines that identifies a M_r 24,000 protein present on null-ALL and B-CLL cells (24). A monoclonal antibody (PI 153/3) produced against a neuroblastoma cell line by Kennett and Gilbert (25) was also reported to react against null-ALL, B-CLL, and CML-BC (lymphoid type) (26). The reactivity of NL-22 antibody can be clearly distinguished from that of BA-2 and PI 153/3 antibody by the fact that the NL-22 antigen was not detected in seven cases of B-CLL cells. Thus, of the monoclonal antibodies reported to react with null-ALL, NL-22 antibody shows the greatest specificity. However, tests with a larger panel of normal and malignant cell types will be necessary before claims for the null-ALL specificity of NL-22 antibody can be made. In addition, biochemical characterization of the NL-22 antigen will be important as a way to compare it with cell surface antigens being defined in other laboratories.

Considerable heterogeneity was observed in the surface phenotype of the 16 cases of null-ALL that were typed with the eight monoclonal antibodies developed in this study. Haynes *et al.* (27) also reported the presence of at least five patterns of reactivity by studying surface antigens of T-ALL with an extensive panel of monoclonal antibodies. These findings are con-

Table 5. Absorption analysis of NL-22 monoclonal antibody

Positive absorption	Negative absorption		
Noncultured cells: null-ALL 063, 038, 041, 059, 343, 384, 277 Cultured cell lines: Non-T, non-B origin NALL-1, NALM-6	Noncultured cells: Leukemia cells null-ALL 514, 542 T-ALL 241, 247 AML 045, 051, 089 545, 550, 563, 564 CML-BC 086 B-CLL 067, 093 Lymphoid cells PBL*: 1, 2, 3, 4 Fetal thymocytes: 2 Spleen cells: 5, 6	Cultured cell lines: Non-T, non-B origin NALM-16, NALM-1, K-562 T-cell origin RPMI 8402, CCRF-HSB-2, 45, MOLT-3, MT-1, P-12 B-cell origin Daudi, CCRF-SB, BALL-1, SK-DHL-2, SK-Ly-16, RPMI 1788 Melanoma SK-MEL-13, SK-MEL-28, SK-MEL-33, SK-MEL-37, SK-MEL-40 Astrocytoma AJ, AS Neuroblastoma SK-NMC, SK-NSH	Renal ca SK-RC-2, SK-RC-6 SK-RC-7, SK-RC-9 Bladder ca T-24 Colon ca HT-29 Cervical ca ME-180

* Peripheral blood lymphocytes.

Table 6. Absorption analysis of NL-1 monoclonal antibody

Positive absorption	Negative absorption	
Noncultured cells:	Noncultured cells:	Cultured cell lines:
null-ALL	Leukemia cells	Non-T, non-B origin
063, 038	null-ALL	NALM-16
041, 349	514	T-cell origin
Cultured cell lines:	T-ALL	CCRF-HSB-2
Non-T, non-B origin	241, 247	45
NALL-1	AML	MOLT-3
NALM-6	045, 089	B-cell origin
T-cell origin	563, 564	CCRF-SB
RPMI 8402	CML-BC	BALL-1
CCRF-CEM	086	RPMI 1788
B-cell origin	B-CLL	Oda
Daudi	082	RPMI 8226
Raji	Lymphoid cells	Melanoma
SK-DHL-2	Fetal thymocytes	SK-MEL-37
SK-Ly-16	2	Neuroblastoma
	Spleen cells	SK-NSH
	5, 6	Renal ca
		SK-RC-7

sistent with the current view that the surface phenotype of leukemia cells reflects the phenotype of the normal precursor, and that leukemic transformation can occur at a number of different steps in the differentiation pathway. It is possible, however, that malignant transformation modifies expression of cell surface antigens. The phenotype of leukemia cells might not correspond, therefore, to a normal cell counterpart, and the heterogeneity observed with leukemias may not reflect a corresponding diversity in the normal physiological pathway of differentiation.

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